



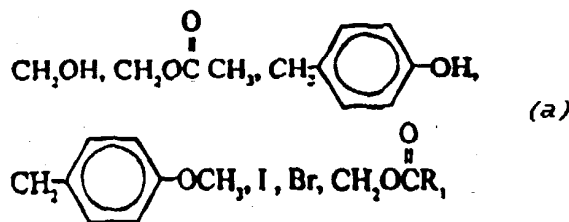
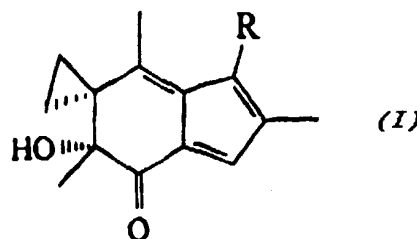
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: ACYLFULVENE ANALOGUES AS ANTITUMOR AGENTS

## (57) Abstract

A method of inhibiting tumor cell growth in a subject is provided comprising contacting the tumor with a therapeutic amount of an acylfulvene analog having structure (I), wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein R is (a), where R<sub>1</sub> = alkyl, aryl, NH<sub>2</sub>, NHR, or NR<sub>2</sub>.



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## ACYLFULVENE ANALOGUES AS ANTITUMOR AGENTS

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15

BACKGROUND OF THE INVENTION

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Multiple agent chemotherapy has curative potential in some hematologic malignancies and advanced rapidly proliferating solid tumors. Curative chemotherapy has benefitted from the discovery of new, relatively non-cross resistant agents, and more effective use of existing agents. Interventions which increase the efficacy of conventional agents include more effective regimens for multiple drug administration, minimization of drug toxicities and increased use of adjuvant, surgical or radiation therapy.

30

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Despite recent advances, patients with many types of malignancies remain at significant risk for relapse and mortality. After relapse, some patients can be reinduced into remission with their initial treatment regimen. However, higher doses of the initial chemotherapeutic agent or the use of additional agents are frequently required, indicating the development of at least partial drug resistance. Recent evidence indicates drug resistance can develop simultaneously to several agents, including ones to which the patient was not exposed. The development of multiple-drug resistant (mdr) tumors may be a function of tumor mass and constitutes a major cause of treatment

failure. To overcome this drug resistance, high-dose chemotherapy with or without radiation and allogenic or autologous bone marrow transplantation is employed. The high-dose chemotherapy may employ the original drug(s) or  
5 be altered to include additional agents. The feasibility of this approach has been demonstrated for hematopoietic and solid tumors. The development of new drugs non-cross resistant with mdr phenotypes is required to further the curative potential of current regimens and to facilitate  
10 curative interventions in previously treated patients.

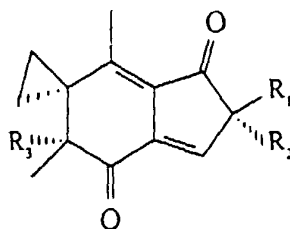
Recently, the in vitro anti-tumor activity of a novel class of natural products called illudins was examined in Kelner, M. et al., *Cancer Res.* 47:3186 (1987),  
15 incorporated herein by reference. Illudin S and M are two types of illudins known to exist. Illudins have a chemical structure entirely different from other chemotherapeutic agents. Illudin compounds were previously purified and submitted for evaluation to the National Cancer Institute  
20 Division of Cancer Treatment (NCI DCT) in vivo drug screening program but had a low therapeutic index in other experimental tumor systems in accordance with NCI studies. The extreme toxicity of illudins has prevented any applications in human tumor therapy.

25 Thus, there exists a need for chemotherapeutic agents which are toxic to tumors, and especially mdr tumors, and have an adequate therapeutic index to be effective for in vivo treatment. The subject invention  
30 satisfies this need and provides related advantages.

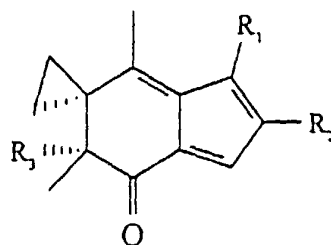
#### SUMMARY OF THE INVENTION

A method of inhibiting tumor cell growth in a  
35 subject is provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure:

3



or



wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

$R_1$  is an alkyl or hydrogen;

$R_2$  is an alkyl; and

$R_3$  is an alcohol or ester.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the sensitivity of breast carcinoma and myeloid leukemia cells versus other tumors to illudin S.

Figure 2 shows the active sites of illudin S.

Figure 3 shows an NMR spectrum demonstrating the presence of a short acting intermediate in acid. Signal A is from the hydrogen on the double bond in the 5 membered ring (illudin M). Signal B is from the hydrogen atom on the short lived intermediate that results from the cyclopropane ring opening up (but before the double bond reacts). Signals marked at C are from the product that

results when the double bond has reacted. With time, the signal peaks from illudin M will disappear and the peaks at position C will be the predominate signals. Signal B will disappear concurrently with Signal A confirming it is a short lived intermediate arising from illudin M.

Figure 4 shows the effect of illudin S on Molt-4 tumor growth in athymic mice (Balb/c).

Figure 5 shows the effect of dehydroilludin M on tumor growth.

Figure 6 shows the response of HL60/MRI xenograft to dehydroilludin M.

Figure 7 shows illudin S uptake using relatively sensitive HL60 cells and resistant B cells.

Figure 8 shows the rapid intracellular accumulation of illudin S by HL60 cells was saturated at high concentrations.

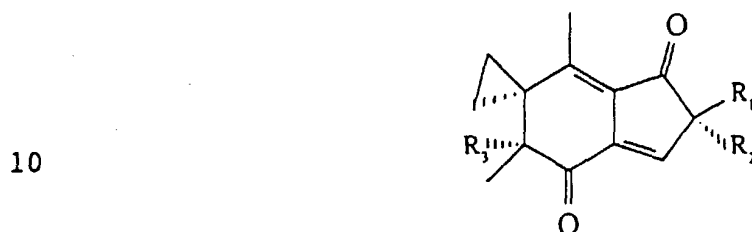
Figure 9 shows the analysis of the initial uptake of illudin S by HL60 cells at varying concentrations conformed to Michaelis-Menton saturation constants.

Figure 10 shows the effect of 6-hydroxymethylacylfulvene and other conventional anticancer agents on human lung adenocarcinoma MV522.

Figure 11 shows the effect of different doses of 6-hydroxymethylacylfulvene on human lung adenocarcinoma MV522.

DETAILED DESCRIPTION OF THE INVENTION

A method of inhibiting tumor cell growth in a subject is provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure:



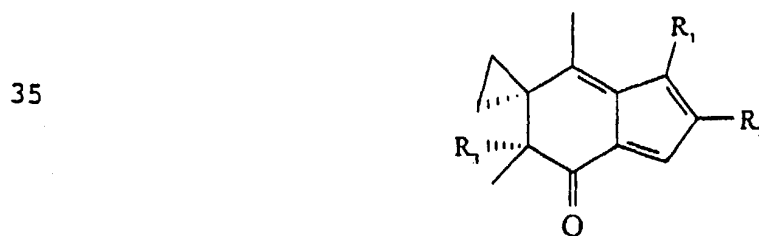
wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

R<sub>1</sub> is an alkyl or hydrogen;  
 R<sub>2</sub> is an alkyl; and  
 R<sub>3</sub> is an alcohol or ester.

20 The analog can be any compound having the stated structure. Examples of the effective analogs are:



A method of inhibiting tumor cell growth in a subject is also provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure:



wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

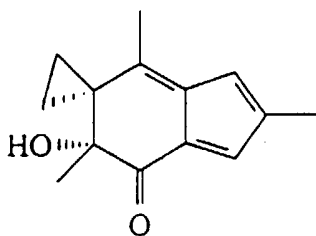
$R_1$  is an alkyl, alkoxyl or hydrogen;

5  $R_2$  is an alkyl; and

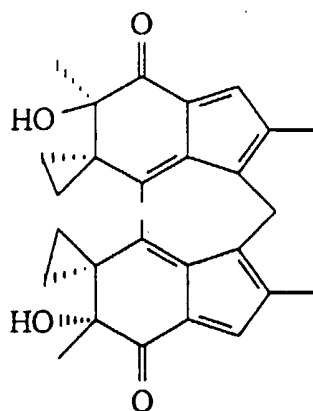
$R_3$  is an alcohol or ester.

The analog may be any compound having the stated structure. Two examples of effective analogs are:

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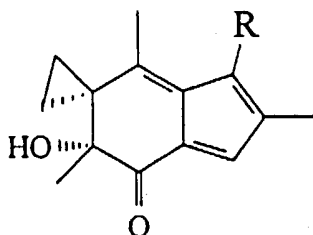
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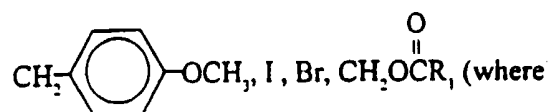
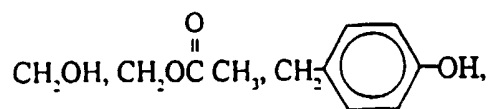
The analog may also be an acylfulvene analog having the structure:

25



30 wherein R is

35



$R_1$  = alkyl, aryl,  $NH_2$ ,  $NHR$ , or  $NR_2$ .



By "inhibiting" is meant either decreasing the tumor cell growth rate from the rate which would occur without treatment or causing the tumor cell mass to decrease in size. Inhibiting also includes a complete regression of the tumor. Thus, the analogs can either be cytostatic or cytotoxic to the tumor cells.

The subject can be any animal having a tumor. The analogs are effective on human tumors in vivo as well as on human tumor cell lines in vitro.

The tumor can be contacted with the analog by any effective means, many of which are well known in the art. The route of administration to the subject can include intravenous, oral, intraperitoneal, and oral and nasal inhalation. The preferred route of administration depends on the subject and type of tumor encountered.

Applicants have made the surprising discovery that analogs of illudin S and M can be made which are less toxic than illudin S and M but are a more effective chemotherapeutic agent in vivo. As noted above, illudin S and M have a low therapeutic index due to the extreme toxicity and, therefore, cannot be used therapeutically in humans. Applicants have discovered that various modifications in illudin S and M inhibit nucleophiles from reacting with the compound. This results in less facile opening of the cyclopropane ring and reduces the toxicity of the compound in vivo while resulting in a high therapeutic index. The Applicants have made the further discovery that an acylfulvene analog, 6-hydroxymethylacylfulvene, is markedly more efficacious and nontoxic than previously known S and M illudin analogs.

The various R groups recited define areas which do not affect the vital functional groups of illudins or their analogs and, therefore, can be a wide variety of

substituents. Thus applicants intend that the various R groups recited be construed broadly, for example, alkyl includes any functional group attached to the alkyl group (i.e., an alkylfulvene or the functional group itself).

- 5 The R groups include nitro, halogen, alkyl halide, alcohol, peroxide, endoperoxide, primary, secondary or tertiary amine, ether, ester, sulfhydryl, thioalkyl, thioester, dithioester, disulfide, carboxylic acid, enamine, imine or oxime groups.

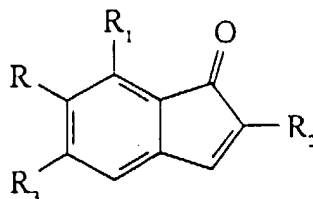
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The therapeutically effective amount of analog varies with the subject. However, it has been found that relatively high doses of the analogs can be administered due to the decreased toxicity compared to illudin S and M.

- 15 A therapeutic amount between 30 to 112,000  $\mu\text{g}$  per kg of body weight has been found especially effective for intravenous administration while 300 to 112,000  $\mu\text{g}$  per kg of body weight is effective if administered intraperitoneally. As one skilled in the art would  
20 recognize, the amount can vary depending on the method of administration. Further, the amount can vary if the analog is linked to a toxin.

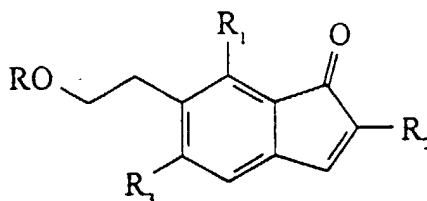
- The analogs can be attached to a reagent to form  
25 a complex which binds to a tumor-associated antigen. Such methods are well known in the art and can include a linker which serves to connect the reagent to the analog. Such attachment can include any chemical bond, as for example a covalent bond. The reagent can be any reagent which  
30 specifically binds to a tumor-associated antigen on the tumor cell or in the tumor cell area. Typically such reagent is an antibody, either polyclonal or monoclonal. These complexes can then be used in therapy. The methods of the invention can be practiced on any tumor cells but  
35 are especially effective against tumor cells of myeloid, epidermoid, T-cell leukemia, and lung, ovarian and breast carcinoma.

Also disclosed is a compound having the structure:



wherein R = methyl; and R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> = methyl or alkyl.

Also disclosed is a compound having the structure:



R = H or methyl; and R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> = methyl or alkyl.

#### EXAMPLE I

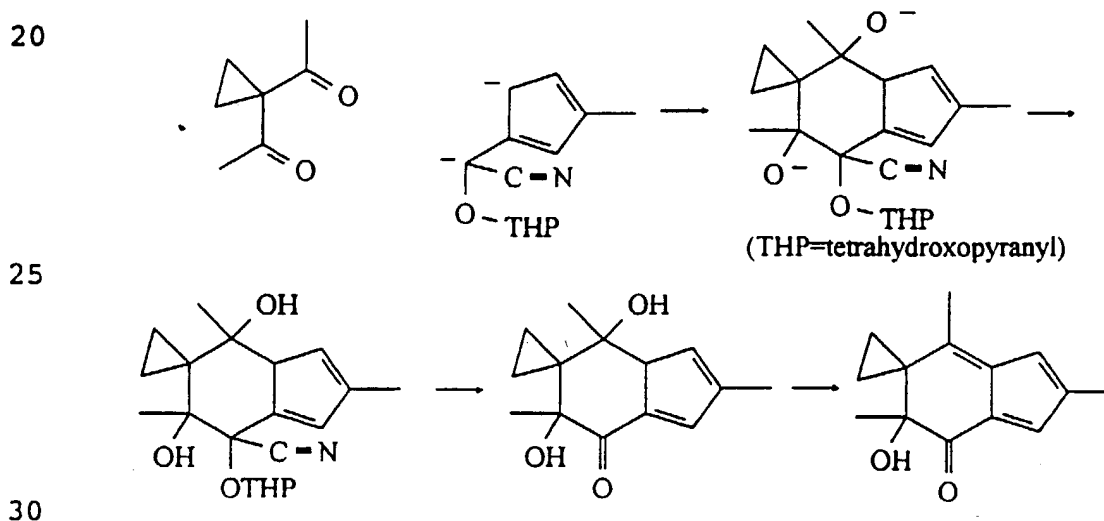
##### Synthesis of Dehydroilludin M

A mixture of illudin M (200 mg) and pyridinium dichromate (1 g) in dry dichloromethane (60 ml) was stirred at room temperature in a flask equipped with a rubber septum so that an atmosphere of argon could be maintained. After 20 hours, the reaction mixture was diluted with diethyl ether (20 ml) and filtered through a short column of silica gel. The column was further eluted with more diethyl ether and the combined filtrate was concentrated, giving a residue which was chromatographed on silica gel with hexane-ethyl acetate (10:1) as eluent. The desired compound was obtained in early fractions from the chromatography. The yield was 140 mg of white crystals melting at 64-65°C. NMR spectral data were recorded for this compound.

**EXAMPLE II**  
**Synthesis of Fulvene**

Illudin S (50 mg) was dissolved in water (2 mL)  
 5 and 3N hydrochloric acid (2 mL) added to the solution. The  
 resulting solution soon became cloudy (within 30 min) and  
 a yellow precipitate formed. The mixture was placed in the  
 refrigerator overnight; then it was extracted with  
 chloroform (10 mL). The yellow chloroform solution was  
 10 dried ( $\text{MgSO}_4$ ) and the solvent was removed under reduced  
 pressure leaving an orange-yellow gum. This material was  
 chromatographed on silica gel with hexanes : ethyl acetate  
 (6 :1) as eluent giving the fulvene (20 mg) and the  
 bisfulvene (10 mg). NMR spectral data were recorded for  
 15 these compounds.

Alternatively, a total synthesis of the fulvene  
 can also be achieved in the following way:



Reaction of the known 1, 1-diacetyl cyclopropane  
 with the dianion of the cyclopentadiene derivative shown  
 gives a diol which on mild acid treatment gives the  
 diolketone. Selective elimination of a tertiary hydroxyl  
 35 group gives the desired fulvene.

## EXAMPLE III

In Vitro Studies

To assess cytotoxic effects, various concentrations of illudins were added to cultures of cells for 48 hours, then cell growth/viability was determined by trypan blue exclusion. As an alternative to 48 hour continuous exposure studies, cells were plated in liquid culture in 96 well plates, exposed to various concentrations of illudins for 2 hours, pulsed with [<sup>3</sup>H]-thymidine for one to two hours and harvested onto glass filters. The filter papers were added to vials containing scintillation fluid and residual radioactivity determined in a beta (scintillation) counter.

15

When screening the sensitivity of other solid tumor cell lines to illudin S, a breast cell line, MCF-7, was noted to be markedly sensitive (Figure 1). Another breast cell line maintained in our laboratory, MDA-231, was also found to be markedly sensitive to illudin S (Figure 1).

Studies with dehydroilludin M indicated this analog also displayed selective toxicity towards myeloid leukemia cells and breast carcinoma lines MCF-7 and MDA-231 (Table 1).

25

TABLE 1

5 Histiospecific cytotoxicity of illudin S and dehydroilludin M as demonstrated by inhibition of thymidine after a two hour exposure to the toxins (N - 3).

10	<u>Compound</u>	<u>Illudin S</u>	<u>IC<sub>50</sub>(nM/L)</u>	
			<u>Dehydroilludin M</u>	
	HL60, myeloid	7 ± 1	246 ± 19	
	8392, B-cell	236 ± 31	> 38,000	
	8402, T-cell	669 ± 196	> 38,000	
15	242, melanoma	607 ± 70	> 38,000	
	547, ovarian	607 ± 110	> 38,000	
	SL-2, murine (thymic)	142 ± 15	5,235 ± 277	
	MCF-7, breast	58 ± 5	653 ± 65	
20	MDA-231, breast	2.0 ± 0.2	112 ± 17	

Because previous studies showed that CEM *mdr* variants were not resistant to illudin S, several other *mdr* cell types were studied for susceptibility to illudin S and the dehydroilludin M. These *mdr* daughter cell lines demonstrate a 200 to 800 fold increase in resistance to multiple conventional chemotherapeutic agents, but showed minimal or no resistance to illudin S or dehydroilludin M (Table 2). Thus, *mdr* cells associated with or without the gp170 protein were still susceptible to illudin toxicity. These studies indicate that illudins' novel structure confers relative non-cross resistance in multidrug resistant hematopoietic cell lines. The derivative of illudins, dehydroilludin M, is slightly less toxic than the parent illudin compound, but results (Table 2) indicate that there is no cross-resistance to this compound in various *mdr* cell lines.

The effect of illudin S and dehydroilludin M on L1210, murine bone marrow CFU-gm, and C1498 (AML cell line) was studied. Illudin S was the most potent agent ever tested in this assay and displayed the largest differential effect ever noted between L1210 and AML leukemia lines and CFU-gm zone cites (Table 3). The derivative,

dehydroilludin M, while less toxic was markedly more selective towards the AML line. It inhibited AML colony formation at concentrations where it had no effect on the CFU-gm cells (Table 4).

5

TABLE 2

Sensitivity of Different Mdr Lines to Illudin S

10

<u>MDR cell line available</u>		<u>Illudin S</u>	<u>Dehydroilludin M</u>
15	CEM Variants Parent	8.3 $\pm$ 2.6	nt
	VM-1	16.2 $\pm$ 6.4	nt
	AraC	14	nt
	VLB100(gp170+)	3.7 $\pm$ 0.7	nt
	Dox (gp170+)	14	
20	MDA-231(Breast) Parent	0.85 $\pm$ 0.23	54 $\pm$ 7
	3-1(gp170+)	0.89 $\pm$ 0.38	58 $\pm$ 11
25	MCF7-wt(Breast) Parent	0.88 $\pm$ 0.11	92 $\pm$ 15
	ADR (GSH-transferase)	3.7 $\pm$ 0.4	68 $\pm$ 15
	HL-60 Parent	3.1 $\pm$ 1.1	163 $\pm$ 11
	ADR (gp150+)	1.9 $\pm$ 0.8	191 $\pm$ 44
30	KB variant Parent	0.58 $\pm$ 0.12	125 $\pm$ 14
	C-1 (gp170+)	0.69 $\pm$ 0.15	80 $\pm$ 18
	VEL(gp170+)	0.69 $\pm$ 0.11	78 $\pm$ 19
35	L1210 Parent	0.42 $\pm$ 0.08	62 $\pm$ 8
	DDPt(cis-plat)	0.46 $\pm$ 0.12	119 $\pm$ 39
	BCNU	0.58 $\pm$ 0.08	100 $\pm$ 31
	PAM(melphalan)	0.62 $\pm$ 0.15	73 $\pm$ 31
	CPA(cyclophos)	0.46 $\pm$ 0.12	38 $\pm$ 15

40

TABLE 3

Inhibition of Growth by Illudin S

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<u>Illudin S Concentration</u> (ug/disc)		<u>Zone of Inhibition</u>		
		<u>L1210</u>	<u>Go</u>	<u>Colon 38</u>
50	2.50	500	240	30
	1.25	400	70	0
	0.63	320	30	0

TABLE 4

5 Effect of Illudins on Colony Formation

10	<u>Compound</u>	<u>Dilution</u>	<u>Zone Size</u>		
			<u>L1210</u>	<u>CFU-GM C1498 (AML)</u>	
15	Illudin S	1/1,000	850	400	> 1000
		1/4,000	600	200	800
		1/16,000	550	0	550
		1/64,000	300	0	250
20	Dehydroilludin M	1/25	400	200	> 1000
		1/125	200	100	750
		1/125 (repeat)	300	50	700
		1/625	100	0	400

## EXAMPLE IV

Structure Function Studies

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The structure-function studies were performed by synthesizing derivatives of the illudins and examining their in vitro toxicity for HL60 leukemia cells (Table 5). This study identified three critical sites for illudin toxicity. These include the cyclopropane ring (site A), the alpha/beta unsaturated bond site (site B), and the ketone group (site C) (Figure 2). Alteration of any of these sites resulted in up to a 4 log decrease in toxicity. In contrast, the non-ring primary hydroxyl group (Figure 2, site D) does not contribute to toxicity. Various large chemical groups can be attached to this site without altering toxicity. Many of the derivatives with a marked decrease in toxicity (as compared to illudin S or M) are still more potent than conventional chemotherapeutic agents such as BCNU or cis-platinum (Table 5).



TABLE 5

5                    IC<sub>50</sub> for Various Illudin Derivatives Versus  
                         Other Agents in HL-60 cells

	<u>COMPOUNDS</u>	<u>nM</u>
10	Illudin S or M	10
	Dihydroilludin S or M	100,000
	Acylfulvene	500
	Dehydroilludin M (diketone)	246
15	Isoilludin M	3,800
	Ptaquiloside	7,700
	Pterosin C	12,500
	2,5,6,7-tetramethylindenone	475
	Illudin tosylate	38
20	DNA polymerase inhibitor: Aphidocolin	2,100
	Alkylating agent: BCNU	23,300
	Crosslinking agent: cis-platinum	550 ±14
	Alkylating agent: MNNG	15,000
25	Protein Synthesis Inhibitor: Ricin	0.2

## EXAMPLE V

Structure-Function Studies: Chemical

30

Illudin M is readily converted to stable aromatic compounds (on treatment with dilute HCl) which in cell culture studies are more than 1,000 fold less toxic. The chlorine-carbon bond formation, cyclopropane ring opening and extrusion of the tertiary hydroxyl (as water) are synchronous. The intermediate formed can be detected by NMR spectroscopy of the reaction mixture (Figure 3). The intermediate, however, is highly reactive and is quickly converted to a phenol by attack of a second nucleophile, i.e., water. Thus, under acidic conditions, illudin M is clearly bifunctional.

The above studies indicate that the toxicity of illudins is related to the ease with which the tertiary hydroxyl can be removed and the cyclopropane ring opened. It was found that illudin toxicity depends on the combined

effects of the cyclopropane group (site A, Figure 2), the two double bonds (conjugated diene) (site B), and the ketone (site C). It was hypothesized that oxidation of the secondary hydroxyl group in the five membered ring to a ketone would alter the potency or selectivity of the molecule by contributing to further electron delocalization within the molecule. The new ketone group acts as an "electron sink" so that electrons of the cyclopropane C-C bonds are delocalized towards the ketone rather than to the carbon atom bearing the tertiary hydroxyl. This means the incipient carbocation, forming as the carbon-oxygen (oxygen of the tertiary hydroxyl) bond breaks, is not as stable as in the case of illudin M. Therefore, carbon-oxygen bond breaking is less favorable and reactivity is reduced. This ketone derivative, termed dehydroilludin M, was synthesized and was less toxic to HL-60 cells in vitro than illudin S or M (Table 4). As discussed above, the toxicity of dehydroilludin M appeared relatively selective for myeloid and breast carcinoma cells in vitro (Figure 1 and Table 1).

20

Consistent with the above hypothesis are the results of the kinetics of the reaction of illudin M and dehydroilludin M with dilute HCl. In dilute HCl, illudin M undergoes a pseudo first-order reaction ( $k = 4.7 \times 10^{-3} \text{ min}^{-1}$ ,  $t_{1/2} = 148 \text{ minutes}$ ). Dehydroilludin M also demonstrated first-order kinetics but the reaction was considerably slower ( $k = 2 \times 10^{-4} \text{ min}^{-1}$ ,  $t_{1/2} = 2765 \text{ min}$ ). In the reaction with dehydroilludin M, no intermediate could be detected by NMR spectroscopy. Presumably it formed too slowly and is too short-lived to be detected. The lower reactivity shown by dehydroilludin M suggests it is more selective in its reaction with nucleophiles and thus has a lower toxicity compared to illudin M.

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The reaction of illudins with a naturally occurring nucleophile, glutathione has also been studied. At a wide pH range, from pH 3 to pH 9, glutathione

spontaneously reacts with illudin M, illudin S, or dehydroilludin M, producing products analogous to those from the reaction of illudin M and HCl. The reaction rate is optimized at a pH of 6.1 to 7.0, indicating the reaction  
5 could occur intracellularly.

The toxicity of illudins towards a breast cell carcinoma line MCF7-wt and its MDR resistant daughter line MCF/Adr was then studied. The gp170 negative daughter cell  
10 line is drug resistant on the basis of a 50 fold increase in glutathione transferase, which results in a 200 to 800 fold decrease in sensitivity to conventional chemotherapeutic agents. This line also shows a 4.1 fold decrease in glutathione content. This daughter line showed  
15 a 4.2 fold decrease in sensitivity to illudin S (parent IC<sub>50</sub> 0.88 nmoles/l; daughter line 3.70 nanomoles/l) versus the 200 to 800 fold seen with other agents. Kinetic studies on the ability of illudins to inhibit glutathione transferase indicated there was no direct inhibition of enzyme  
20 activity. These findings show that while illudin toxicity is inversely correlated with intracellular glutathione content it is not correlated with glutathione transferase activity.

25

#### EXAMPLE VI

##### Animal Studies

Using procedures set forth in Leonard, J.E. et al., *Cancer Res.* 47:2899-02 (1987) and Dillman, R.O. et al,  
30 *Cancer Res.* 45:5632-36 (1985), both incorporated by reference herein, Molt-4 (human T-cell leukemia) xenografts were established in four week old athymic Balb/c nu/nu mice. After 3 weekly doses of total body radiation (600 cGy), mice were given subcutaneous flank injections of  
35 Molt-4 cells together with irradiated (6000 cGy) HT-1080 feeder cells. Two animals received only irradiated HT-1080 feeder cells to ensure these cells did not induce tumors.

Animals were monitored for Molt-4 tumor development and when tumors were palpable (approximately 4 x 4 mm at 5 to 7 days), mice were randomized into groups of 5 as previously described. Control mice received intraperitoneal saline and treated mice received either 300  $\mu\text{g}/\text{kg}$  illudin S, 30 or 300  $\mu\text{g}/\text{kg}$  dehydroilludin M, IP twice weekly. In mice given illudin S there was tumor growth delay (Figure 4).

In contrast, in nude mice which received the dehydroilludin M at the low dosage of 30  $\mu\text{g}/\text{kg}$  (the compound was subsequently found to be nontoxic to mice at 60,000  $\mu\text{g}/\text{kg}$  IP twice a week), three of five tumors underwent complete regression, but two tumors failed to respond (figure 5). The two apparently resistant tumors were harvested and tested in vitro for resistance to illudin S and dehydroilludin M. There was no evidence of resistance to either compound. Two of the complete responders were followed for over twelve weeks without evidence of tumor regression.

Using a different source of athymic nude mice, these experiments were repeated. In these studies there was little effect of illudins on tumor growth. The reason for this variability in response to Molt-4 xenografts probably relates to the low doses of dehydroilludin M, interanimal variations in glutathione metabolism, or drug distribution.

The efficacy of dehydroilludin M was then screened in a syngeneic model using murine SL-2 cells. SL-2 leukemia/lymphoma cells are injected subcutaneously and metastasized to lymph nodes, spleen, and lungs, and drug efficacy in this model is determined by increased life span (ILS). The SL-2 cells were administered at 2.5 million cells per animal and treatment was delayed for 7 days until the tumors were palpable. This is a relatively stringent

test against established tumors and contrasts to general drug screens in the SL-2 model which normally use only 0.5 million cells and starting drug treatment at 3 days. Dehydroilludin M had a little effect at 30 mg/kg IP twice a week, ILS 5%, and 60 mg/kg IP twice a week, ILS 18%. When administered IV at 0.03 mg/kg, twice a week, the ILS increased to 38%. This suggests the drug is metabolized by the liver and is likely more efficacious when administered IV.

10

During the course of these in vivo experiments, it became clear from in vitro experiments, that histiospecificity of illudins depends upon the presence of an active energy-dependent pump. The SL-2 and the Molt-4 cells were studied and it was determined that the uptake mechanism was not present. Therefore, the studies were redirected into xenograft models that used cells of myeloid lineage.

20

Human HL-60 cells capable of growing as xenografts in nude mice without animal radiation were obtained from Dr. Theodore Brightman (NCI). These cells termed HL-60 MRI cells, were confirmed to have energy-dependent uptake pump, a not unexpected finding as their parental cells possess the pump. Dehydroilludin M induced dose related tumor inhibition when administered IP on a twice a week schedule (figure 6). The MTD IP dose for dehydroilludin M was reached in these studies on the 2 dosages per week IP dose schedule. Similar tumor regressions have been observed with IV dehydroilludin M.

30

In collaboration, the in vivo effects of dehydroilludin M was again studied. Initially the compound was studied against L1210 cells. A dose of 2.5 mg/kg IP given daily for 5 days resulted in an ILS of only 9%. The dehydroilludin M was then administered as a 24 hour infusion (5.0 mg/kg); the ILS was 11%. After we became

35

aware of the presence of the energy-dependent uptake in human myelocytic cells, dehydroilludin M was screened for in vivo efficacy against a syngeneic mouse AML model using C1498 cells and a single bolus of illudin S, 2.5 mg/kg IP, produced an ILS of 35%. A second trial using the same dosage, administered IP once a day for 5 days resulted in a 44% ILS. As the animals can tolerate 60 mg/kg IP or 1 mg/kg IV (tail vein) on a twice a week schedule for 4 weeks without demonstrating weight loss or a decrease in food/water intake, it is possible to further optimize both dosage and treatment schedule.

#### EXAMPLE VII

##### HL60/MRI Mouse Experiment With Acylfulvene and Dehydroilludin M

Thirty mice were injected subcutaneously, over the shoulder, with 500,000 HL60/MRI cells (human myeloid leukemia tumor cells). Treatment was begun on day 11, rather than immediately. This delay in starting treatment is a stringent test to determine whether a compound is effective. By delaying treatment, the tumor cells become firmly established.

The mice were divided into 6 groups of 5 each. One group was the control and these animals received on a placebo, the solution used to dilute the agent. The other groups received the following compounds and dosages: the dehydroilludin M compound at 1.0 mg/kg, the dehydroilludin M at 3.0 mg/kg, the Acylfulvene at 0.3 mg/kg, the Acylfulvene at 1.0 mg/kg, the Acylfulvene at 3.0 mg/kg. All animals received the placebo or drugs by intravenous injection using a tail vein. The placebo or drugs were administered on a twice a week schedule.

Results are summarized in the accompanying table 6. Both the dehydroilludin M and the Acylfulvene compound were effective at inhibiting tumor growth and demonstrated dosage dependence inhibition (the more drug administered, the less the tumors grew). The animals receiving the highest amount of either drug did not display any evidence of adverse effect, such as a decrease in food or water intake, nor a statistically significant decrease in body weight. These results show that higher dosages of either drug can be administered. Also, that the drug could be administered on a more effective dosage schedule, such as on a daily basis.

TABLE 6

Summary: HL60/MRI experiment, intravenous - # 1

		<u>BY TOTAL TUMOR WEIGHT [Mg]</u>				
		<u>DAY 11</u>	<u>DAY 18</u>	<u>DAY 25</u>	<u>DAY 32</u>	<u>DAY 40</u>
<u>CONTROL</u>						
25	No Drug	99±36	845±282	3299±1080	10162±4123	16747±5061
<u>Dehydroilludin M</u>						
30	1 mg/kg IV	114±55	883±311	2274±992	6025±1772	11507±3707
	3 mg/kg IV	101±40	911±309	2127±1092	2854±1260	4784±2303
35	<u>Acylfulvene</u>					
	0.3 mg/kg IV	73±38	540±167	1352±520	3204±1147	9501±4605
40	1 mg/kg	58±32	582±297	964±685	2321±1434	6275±2865
	3 mg/kg	38±30	369±250	336±215	437±238	1201±501

## EXAMPLE VIII

General In Vitro Screening Procedures and  
Cell Uptake Studies

5           In keeping with the suggestions of the previous examples and our concentration on mechanisms of illudin action and tissue specificity, other myeloid leukemia cell lines can be screened for rapid illudin uptake (KG1, KG1a, HEL, K562, OCI-M1, AML-193).

10

          The procedures for in vitro screening of illudin compounds are detailed in the previous examples. Cytotoxicity of new analogs for cell lines is initially evaluated over a 5 log range using growth or semi-solid  
15 colony forming assays, and inhibition of thymidine incorporation. Inhibition of thymidine incorporation is used because earlier studies indicate that thymidine incorporation is preferentially inhibited by illudins and correlates closely with cell death. Analogs are screened  
20 against normal bone marrow progenitors and a variety of cell lines involving various leukemias, B and T cell) and solid tumors (melanoma, ovarian).

In vitro testing of dehydroilludin M on various  
25 cell lines, including MDR lines, can also be performed on DNA-repair deficient cell lines and normal bone marrow progenitors. A variety of other analogs can be prepared. Since these analogs will have alterations in the known active sites, they are expected to result in a similar  
30 tumor inhibition. Screening studies for these analogs can include various mdr cells (to ensure that no cross-resistance occurs) and DNA-repair deficient cell lines.

In vitro testing can also study sensitivity of  
35 other breast cell lines to determine if they are also preferentially sensitive to illudin S, dehydroilludin M, and the fulvene analog.



## EXAMPLE IX

Assessment of Illudin Uptake in Tumor Cells

While human myeloid tumor cells are sensitive to  
5 illudins, their normal precursors, granulocyte/macrophage  
forming units, are relatively resistant to illudins by 1.5  
to 2.0 logs, demonstrating that the transport system is  
absent from some normal marrow cells and providing a  
therapeutic margin of safety.

10

Specific illudin S uptake was assayed using  
relatively sensitive HL60 cells and resistant B cells. At  
37°C, HL60 myeloid leukemia cells demonstrated rapid uptake  
of illudin S, while the relatively insensitive 8392 B-cells  
15 exhibited comparatively little drug incorporation (Figure  
7). The intracellular accumulation of illudins in the B  
cell line was slow and linear for 7 hours ( $r = 0.984$ ), at  
which time the intracellular concentration approached that  
of the incubation mixture. HL60 cells, in contrast,  
20 rapidly accumulated the toxin and intracellular  
accumulation reach a plateau within one hour. HL60 cells  
exposed to 10 nM illudin S concentrated the toxin 19 fold,  
whereas B cells did not actively concentrate the toxin.  
The rapid intracellular accumulation of illudin S by HL60  
25 cells was saturated at high concentrations (Figure 8). In  
contrast, illudin S accumulation in 8392 B cells remained  
concentration dependent. Analysis of the initial uptake of  
illudin S by HL60 cells at varying concentrations revealed  
that the influx of illudin S conformed to Michaelis-Menton  
30 saturation kinetics (Figure 9). The  $V_{max}$  for HL60 cells  
was 27 picomoles/minute/mg of protein and the  $K_m$  was 4.2  
 $\mu M$ . This indicates HL60 cells have a very high transport  
capacity for illudins as the  $V_{max}$  for illudins is 5 times  
the  $V_{max}$  for folate, a vitamin required by cells.

35

Cold (4°C), 1% azide, and the metabolic blockers 2-deoxyglucose and antimycin A, all block uptake of illudin S into HL60 cells but have little effect on the insensitive 8392 B-cells (Table 7). These studies indicate that illudin S is transported and concentrated into HL60 cells by an energy dependent transport system, whereas the transport into insensitive B-cells occurs only by diffusion (passive or nonenergy requiring transport). MCF7 breast tumor cells also demonstrated inhibition of uptake by cold. The finding of an energy-dependent transport mechanism explains why myeloid and breast tumor cells are so sensitive to illudins with short exposure times, but B-cells are not.

15

TABLE 7

Uptake of [<sup>3</sup>H] Illudin S by HL60 Myeloid versus 8392 B-cells

20

Maximum uptake per hour (picomoles)\*

<u>Conditions</u>	<u>HL60</u>	<u>8392</u>	<u>MCF7</u>
37°C	75 ± 16 <sup>b</sup>	5.5 ± 1.4	29 ± 4
4°C	4.3 ± 0.9	3.4 ± 1.0	4.0 ± 2.1
1% Azide	8.7 ± 1.4	4.3 ± 1.3	NT <sup>b</sup>
2-deoxyglucose & Antimycin A	16.7 ± 3.5	3.6 ± 1.4	NT

30

Cells were exposed to 100 ng/ml of [<sup>3</sup>H]-labeled illudin S for one hour and harvested as described. Results are expressed as mean ± SE and represent 3 experiments.

\*per 10 million cells

35

<sup>b</sup>NT = not tested

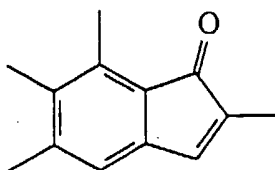
## EXAMPLE X

Synthesis and Structure of 2,5,6,7-  
Tetramethyl-1-Indenone and Dehydropterosin Compounds

5 First 2,4,5,6-tetramethyl-1,3-indanione was synthesized by preparing a solution of 1,2,3-trimethylbenzene and methylmalonylchloride in carbon disulfide and adding aluminum trichloride dropwise over two hours. The mixture was refluxed for 2 more hours, crushed  
10 ice added, and extracted three times with chloroform. The combined extract was washed with brine, dried, and solvent removed to leave a residue which was purified by chromatography with 1% ethyl acetate in benzene. Removal of solvent and purification by sublimation gave the desired  
15 product.

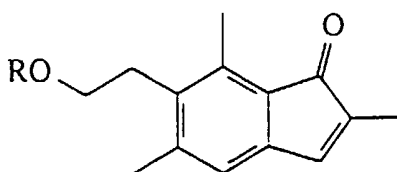
The 2,5,6,7-tetramethyl-1-indenone was prepared by reducing 2,4,5,6-tetramethyl-1,3-indanione with zinc dust at 50°C. Product was purified by chromatography with  
20 1% ethyl acetate in benzene to yield two isomers. The major isomer was treated with 10% potassium hydroxide, then purified by sublimation. The compound has the structure:

25



Dehydropterosin O synthesis: 3-acetoxy-6(beta-methoxy)  
30 ethyl-2,5,7-trimethyl-1-indanone was dissolved in tetrahydrofuran and 10% potassium hydroxide and refluxed for two hours. The solution was then extracted three times with ether and the combined extracts chromatographed with 2% ethylacetate in benzene to yield the Dehydropterosin O  
35 compound. The compound has the structure:

26



R = H      Dehydropterosin B

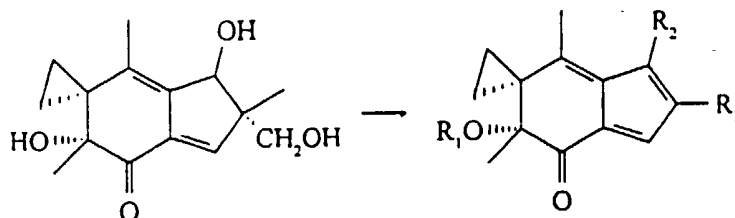
R = CH<sub>3</sub>      Dehydropterosin O

Both compounds were toxic to cells in vitro and have antifungal properties.

#### EXAMPLE XI

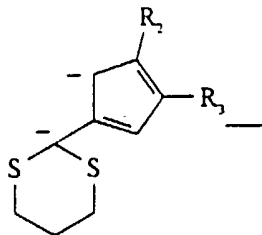
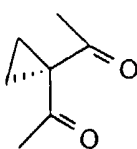
##### Synthesis of Acylfulvene Analogs

Many acylfulvene analogs possessing the key structural features required for antitumor activity may be prepared starting from illudin S or by total synthesis from simple precursors. Illudin S is produced by fermentation of *Omphalotus illudens*. On dissolving this compound in water and adding dilute H<sub>2</sub>SO<sub>4</sub>, it is converted to the acylfulvene (R<sub>1</sub>=H, R<sub>2</sub>=H, R<sub>3</sub>=CH<sub>3</sub>) in 55% yield. A large number of analogs may be obtained from the acylfulvene by modifying the R<sub>2</sub> substituent, e.g., R<sub>2</sub> = hydroxymethyl, bromo, iodo, chloro, fluoro, nitro, p-hydroxybenzyl, p-methoxybenzyl. R<sub>2</sub> can also be polynuclear or heterocyclic aromatic groups.

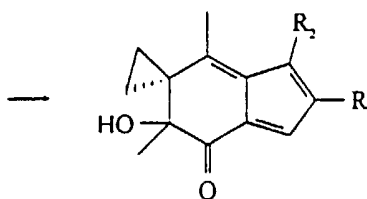
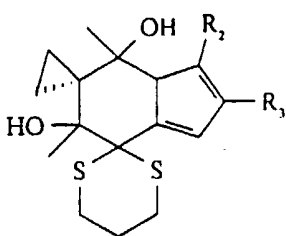


The  $R_1$  group may be acyl or alkyl. Analogs with different  $R_1$  groups (and also  $R_2$  groups) can be prepared by total synthesis as outlined.

5



10



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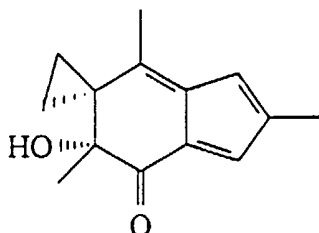
Aldol condensation of 1,1-diacetylcyclopropane with the dianion derived from an appropriately substituted cyclopentadiene gives an intermediate which is readily converted to the acylfulvene. A wide variety of compounds are possible since  $R_1$  and  $R_2$  may be alkyl, aryl or substituted alkyl or aryl groups.

25

**Synthesis of acylfulvene.** Illudin S 2 g (9.2 mmol) was dissolved in 700 mL water followed by addition of 4 M  $H_2SO_4$  (236 mL). The solution was stirred overnight, and extracted with ethyl acetate. The organic phase was washed with saturated  $NaHCO_3$ , water and brine, dried over  $MgSO_4$  and concentrated. Chromatography on silica with hexane and ethyl acetate afforded 0.82 g acylfulvene (50%).  $^1H$  NMR  $\delta$  0.73 to 1.50 (m, 4 H), 1.38 (s, 3 H), 2.00 (s, 3 H), 2.15 (s, 3 H), 3.93 (s, 1 H), 6.43 (s, 1 H), 7.16 (s, 1 H); MS  $m/z$  216 ( $M^+$ ), 202 ( $M^+ - CH_2$ ), 188 ( $M^+ - CH_2CH_2$ ), 173 ( $M^+ - 2CH_2 - CH_3$ ), 170 ( $M^+ - 2CH_2 - H_2O$ ). UV 325 nm ( $8.3 \times 10^3$ ), 235 nm ( $16.6 \times 10^3$ ). The compound has the structure:

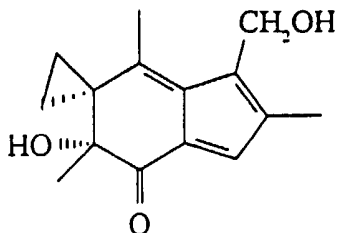
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10                   **Synthesis of 6-hydroxymethylfulvene.** Acylfulvene (550 mg, 2.5 mmol) was dissolved in 40 mL THF and 30% formaldehyde-water solution (40 mL) was added. 4 N H<sub>2</sub>SO<sub>4</sub> solution (26.4 ml) was added to bring the final concentration of H<sub>2</sub>SO<sub>4</sub> to 1 N. The solution was stirred  
15 overnight and extracted with ethyl acetate. The organic phase was washed with saturated NaHCO<sub>3</sub> solution, water and brine and dried over MgSO<sub>4</sub>. Chromatography on silica gel with hexane and ethyl acetate gave 400 mg hydroxymethylfulvene (64%). <sup>1</sup>H NMR δ 0.72 to 1.48 (m, 4 H),  
20 1.38 (s, 3 H), 2.15 (s, 3 H), 2.19 (s, 3 H), 3.90 (s, 1 H), 4.66 (d, J=2.1 Hz, 2 H), 7.10 (s, 1 H). MS m/z 246 (M<sup>+</sup>), 228 (M<sup>+</sup>-H<sub>2</sub>O), 218 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>), 186 (M<sup>+</sup>-CH<sub>3</sub>-CH<sub>2</sub>-CH<sub>2</sub>-OH), 185 (M<sup>+</sup>-H<sub>2</sub>O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>). UV 233 nm (1.0 x 10<sup>4</sup>), 325 nm (7.7 x 10<sup>3</sup>). The compound has the structure:

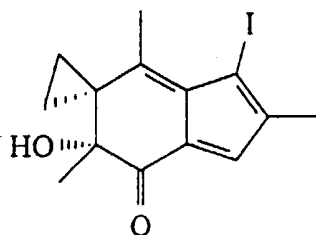
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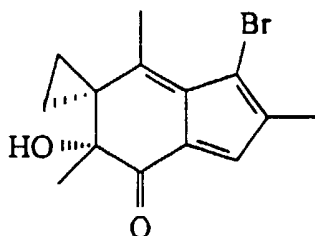
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**Iodofulvene.** To a solution of acylfulvene (60  
35 mg, 0.28 mmol) in 15 ml CH<sub>2</sub>Cl<sub>2</sub>, was added silver trifluoroacetate (63 mg, 0.29 mmol). A solution of iodine (70.5 mg, 0.28 mmol) in 8 mL CH<sub>2</sub>Cl<sub>2</sub>, was added dropwise at 0

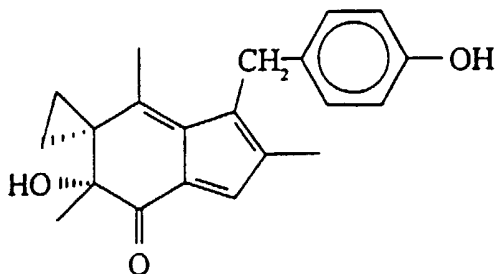
°C. The mixture was stirred at that temperature for 3 hours then filtered through celite and eluted with ether. Concentration of the filtrate gave iodofulvene as a red gum (73 mg, 77%). <sup>1</sup>H NMR δ 0.76 to 1.54 (m, 4 H), 1.38 (s, 3 H), 2.14 (s, 3 H), 2.36 (s, 3 H), 3.87 (s, 1 H), 7.16 (s, 1 H). MS m/z 342 (M<sup>+</sup>), 314 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>), 299 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>-CH<sub>3</sub>), 296 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>-H<sub>2</sub>O), 215 (M<sup>+</sup>-I), 187 (M<sup>+</sup>-I-CH<sub>2</sub>CH<sub>2</sub>), 127 (I<sup>+</sup>). The compound has the structure:



**Bromofulvene.** Acylfulvene (60 mg, 0.28 mmol) was dissolved in 9 ml acetonitrile at 0 °C. N-bromosuccinimide (50 mg, 0.28 mmol) was added and the mixture was stirred at that temperature for 3.5 hours. Water was used to quench the reaction and ether to extract the product. The ether layer was washed with water and brine and dried over MgSO<sub>4</sub>. Chromatography gave bromofulvene as orange crystals (77 mg, 94%; recrystallized from ether acetate-hexane, m.p.. 92-94 °C). <sup>1</sup>H NMR δ 0.75 to 1.55 (m, 4 H), 1.40 (s, 3 H), 2.12 (s, 3 H), 2.33 (s, 3 H), 3.89 (s, 1 H), 7.15 (s, 1 H). MS m/z 295 (M<sup>+</sup>), 293 (M<sup>+</sup>-2), 267, 265, (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>), 252, 250 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>-CH<sub>3</sub>), 249, 247 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>-H<sub>2</sub>O), 215 (fulvene-1). The compound has the structure:



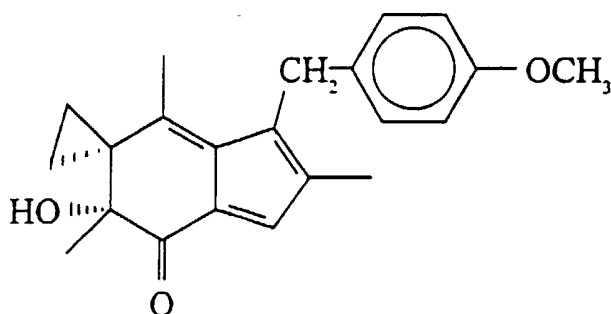
**p-Hydroxybenzylfulvene.** Phenol (40 mg, 0.4 mmol) was added to a solution of hydroxymethyl-fulvene (70 mg, 0.28 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (25 mL). The mixture was cooled to  $-78^\circ\text{C}$  and boron trifluoride etherate (0.3 mL, 2.7 mmol) was added dropwise. The reaction was stirred at that temperature for 1 hour and water was added to quench the reaction. The organic layer was washed with  $\text{H}_2\text{O}$ ,  $\text{NaHCO}_3$ , and brine, and dried over  $\text{MgSO}_4$ . Chromatography on silica gel with hexane-ethyl acetate yielded 90 mg (98%) of red crystals (m. p.  $143\text{--}144^\circ\text{C}$ ).  $^1\text{H}$  NMR  $\delta$  0.59–1.43 (m, 4 H), 1.36 (s, 3 H), 1.76 (s, 3 H), 2.07 (s, 3 H), 3.95 (s, 1 H), 3.97 (d,  $J=7.2$  Hz, 2 H), 4.83 (s, 1 H), 6.74 (d,  $J=8.4$  Hz, 2 H), 6.91 (d,  $J=8.4$  Hz, 2 H), 7.22 (s, 1 H). MS  $m/z$  322 ( $\text{M}^+$ ), 294 ( $\text{M}^+-2\text{CH}_2$ ), 279 ( $\text{M}^+-2\text{CH}_2\text{--CH}_3$ ), 251 ( $\text{M}^+-2\text{CH}_2\text{--CH}_3\text{--CO}$ ), 215, 107. UV 228 nm ( $1.2 \times 10^4$ , with inflections at 243 and 262 nm), 325 ( $7.1 \times 10^3$ ), 410 nm ( $2.6 \times 10^3$ ). The compound has the structure:



**p-Methoxybenzylfulvene.** Anisole (0.04 mL, 0.37 mmol) was added to a solution of hydroxymethylfulvene (10 mg, 0.04 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (5 mL). The mixture was cooled to  $-78^\circ\text{C}$  and boron trifluoride etherate (0.04 mL, 0.36 mmol) was added dropwise. The reaction was stirred at that temperature for 1 hour and water was added to quench the reaction. The organic layer was washed with  $\text{H}_2\text{O}$ , saturated  $\text{NaHCO}_3$ , and brine, and dried over  $\text{MgSO}_4$ . Concentration of the solution gave a residue which was dried in vacuo, yielding the product in quantitative yield (14 mg).  $^1\text{H}$  NMR  $\delta$  0.59–1.40 (m, 4 H), 1.36 (s, 3 H), 1.76 (s, 3 H), 2.07 (s, 3 H), 3.78 (s, 3 H), 3.95 (s, 1 H), 3.99 (d,  $J=16.12$



Hz, 2 H), 6.81 (d, J=8.8 Hz, 2 H), 6.96 (d, J=8.3 Hz, 2 H), 7.22 (s, 1 H). MS m/z 336 (M<sup>+</sup>), 308 (M<sup>+</sup>-CH<sub>2</sub>CH<sub>2</sub>), 215, 121 ((CH<sub>2</sub>-Ph-OCH<sub>3</sub>)<sup>+</sup>). UV 410 nm (2.7 x 10<sup>3</sup>), 325 nm (7.0 x 10<sup>3</sup>), 267 nm (1.0 x 10<sup>4</sup>), 245 nm (inflection), 226 nm (1.9 x 10<sup>4</sup>), 203 nm (1.4 x 10<sup>4</sup>). The compound has the structure:



#### EXAMPLE XII

##### In Vitro Cell Culture Studies of Acylfulvene Analogs

In vitro testing using cell culture assays demonstrated the 6-hydroxymethylacylfulvene, bromoacylfulvene, and iodoacylfulvene analogs were markedly toxic to the target tumor cells HL60 and MV522 at both 2 and 48 hour exposure periods (Table 8 and Table 9). The relative toxicity ratio (2 to 48 hour toxicity) suggested these analogs would be more efficacious in vivo than either the parent Illudin S compound or the analogs described in the original patent application.

TABLE 8

2 hour cytotoxicity of new analogs (as determined by inhibition of thymidine incorporation compared to original analogs and Illudin S

5

2 hour IC50 values (nanomoles/liter)

	<u>HL60 Cells</u>	<u>8392 Cells</u>	<u>MV522</u>
10 Illudin S	10 ± 1	236 ± 22	19 ± 6
Dehydroilludin M	377 ± 81	61,335 ± 9,810	1,826 ± 378
Acylfulvene	998 ± 244	66,435 ± 13,006	727 ± 180
6-hydroxymethylacylfulvene	150 ± 11	7,359 ± 2,096	114 ± 28
15 acetate analog of 6-HMAF	3,333 ± 192	47,455 ± 2,951	1,066 ± 87
bromoacylfulvene	803 ± 88	17,175 ± 890	4,180 ± 424
iodoacylfulvene	2,602 ± 345	10,331 ± 497	956 ± 152
p-hydroxybenzylfulvene	264 ± 38	95,236 ± 11,984	1,180 ± 180
20 p-methoxybenzylfulvene	1,964 ± 84	35,714 ± 7,292	2,045 ± 208

TABLE 9

48 hour cytotoxicity of new analogs compared to original analogs and the parent Illudin S compound.

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48 hour IC50 values (nanomoles/liter)

	<u>HL60 Cells</u>	<u>8392 Cells</u>	<u>MV522</u>
10 Illudin S	3 ± 1	8 ± 2	4 ± 1
Dehydroilludin M	296 ± 66	269 ± 100	313 ± 23
Acylfulvene	364 ± 74	833 ± 152	349 ± 23
6-hydroxymethylacylfulvene	4 ± 1	76 ± 4	73 ± 8
15 acetate analog of 6-HMAF	806 ± 30	4,434 ± 163	486 ± 42
bromoacylfulvene	412 ± 21	1,186 ± 138	356 ± 61
iodoacylfulvene	290 ± 12	1,696 ± 183	556 ± 47
p-hydroxybenzylfulvene	382 ± 39	11,078 ± 388	615 ± 56
20 p-methoxybenzylfulvene	1,051 ± 104	7,143 ± 244	1,548 ± 214

## EXAMPLE XIII

In Vivo Studies

Based on these in vitro screening results, one of the analogs, the 6-hydroxymethylacylfulvene, was chosen for in vivo studies to determine if the analog was indeed more potent than the acylfulvene. The xenograft was again the human lung adenocarcinoma MV522 as it is a nonresponsive model to conventional anti-cancer agents and kills by metastasis (not local tumor invasion). The conventional anti-cancer agents cis-platinum, taxol, mitomycin C, adriamycin, as well as Illudin S were chosen as pharmaceutical controls. A control group was included that received only the solvent used to dissolve the drugs, a 40% dimethylsulfoxide/normal saline mixture (40% DMSO/NS). The 6-hydroxymethylacylfulvene analog actually induced tumor regression in animals. Actual regression of tumors by an anticancer agent has never been noted before in this model. There was no inhibition of tumor growth by conventional anticancer agents or by Illudin S (Figure 10). Animals received only 9 doses of the 6-hydroxymethylacylfulvene analog. There was no evidence of toxic side effects in these animals as evidenced by a decrease in activity, weight gain, food intake, or water intake. There was no significant increase in the life span of cisplatin, taxol, mitomycin C, and Adriamycin treated animals as compared control (DMSO/NS) treated animals. The Illudin S actually caused premature death (drug toxicity) (Table 10). The 6-hydroxymethylacylfulvene treated animals lived significantly longer than controls (DMSO/NS treated), cisplatin, taxol, mitomycin C, and Adriamycin treated animals ( $p < 0.001$  for all groups) (Table 10).

TABLE 10

5

Efficacy of 6-hydroxymethylacylfulvene analog, versus other agents, in the human lung adenocarcinoma MV522 metastatic lung tumor model - First experiment

10

druglife span

controls DMSO/NS IP 3X/WK

100  $\pm$  7%

cis-platinum 3.2 mg/kg IP 1X/WK

102  $\pm$  8%

taxol 4.0 mg/kg IP 5X/WK

100  $\pm$  10%

15 mitomycin C 2.4 mg/kg IP 1X/WK

111  $\pm$  2%

Adriamycin 2.6 mg/kg IP 1X/WK

98  $\pm$  12%

Illudin S 2.5 mg/kg IP 3X/WK

&lt;26%

[5/5 dead at only 3 doses]

20

6-hydroxymethylacylfulvene 10 mg/kg IP 3X/WK 233  $\pm$  18%

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The experiment was repeated with different dosages of the 6-hydroxymethylacylfulvene analog to determine if a dose-response pattern was present. Taxol and low dose Illudin S were again included as pharmaceutical controls (Table 11). A control group

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receiving only the pharmaceutical solvent (40% DMSO/NS) was again included. Tumor regression was again noted at 10 mg/kg, and inhibition of tumor growth was noted with 1 and 5 mg/kg treatment. The taxol and low dose Illudin S again failed to inhibit tumor growth (Figure 11). In this second

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experiment there was again no significant increase in the life span of taxol or Illudin S treated animals as compared controls (40% DMSO/NS treated animals). The 10 mg/kg 6-hydroxymethylacylfulvene and 5 mg/kg 6-hydroxymethylacylfulvene treated animals lived

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significantly longer than controls (DMSO/NS treated),

taxol, or Illudin S treated animals. The probability (or significance) value for 10 mg/kg 6-hydroxymethylacylfulvene treated animals versus the controls, taxol treated, and Illudin S treated animals was less than 0.001 in each case (p < 0.001). The probability value for the 5 mg/kg 6-hydroxymethylacylfulvene treated animals versus the controls, taxol treated, and Illudin S treated animals was also less than 0.001 in each case (p < 0.001). The 1 mg/kg 6-hydroxymethylacylfulvene treated animals also lived significantly longer than the control group (p < 0.01).

TABLE 11

Efficacy of 6-hydroxymethylacylfulvene analog, versus other agents, in the human lung adenocarcinoma MV522 metastatic lung tumor model - Second experiment

<u>drug</u>	<u>life span</u>
controls DMSO/NS IP 3X/WK	100 ± 16% (100% by definition)
taxol 4.0 mg/kg IP 5X/WK	120 ± 10% (not significant)
Illudin S 0.25 mg/kg IP 3X/WK	104% ± 20%
6-hydroxymethylacylfulvene 10 mg/kg IP 3X/WK	232 ± 20%
6-hydroxymethylacylfulvene 5 mg/kg IP 3X/WK	154 ± 13%
6-hydroxymethylacylfulvene 1 mg/kg IP 3X/WK	135 ± 10%

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The experiment was repeated for a third time (Table 12). The amount of taxol administered IP was increased to demonstrate maximum dosage, and a subcutaneous dosage was added due to reports that this route may be more efficacious. Adriamycin and mitomycin C were again included. Two of the new acylfulvene derivatives

(iodoacylfulvene and the p-hydroxybenzylfulvene) were included in this experiment. The diketone analog was included to demonstrate the marked improvement of the 6-hydroxymethylacylfulvene. The 6-hydroxymethylacylfulvene  
5 markedly increased the life span versus controls and all other drug treated animals. The probability (or significance) value for the 6-hydroxymethylacylfulvene versus all the control group, the mitomycin C treated group, the adriamycin treated group, and both of the taxol  
10 treated groups was less than 0.0005 ( $p < 0.0005$ ). This is an extremely significant effect. It is important to note that based on the longevity of 2 particular animals, there is the possibility they may have been cured. Although the diketone also markedly increased the lifespan versus  
15 controls ( $p < 0.002$ ) and other drug treated animals, it was not as effective as the 6-hydroxymethylacylfulvene. Note that the lifespan of the high dose taxol treated animals (6 mg/kg IP) not only decreased below that of the 4 mg/kg taxol treated animals in the previous experiment, but it  
20 was now less than the life span of untreated or control animals. This indicates that the maximum dose for taxol had been reached and drug toxicity was now killing the animals. The other new analogs, the bromoacylfulvene and the p-hydroxybenzylfulvene are also effective in this  
25 model.

TABLE 12

5 Efficacy of 6-hydroxymethylacylfulvene analog, versus other agents, in the human lung adenocarcinoma MV522 metastatic lung tumor model - Third experiment

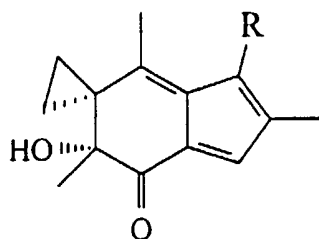
10	<u>drug</u>	<u>life span</u>
	controls DMSO/NS IP 3X/WK	100 $\pm$ 29%
15	taxol 6.0 mg/kg IP 5X/WK	93 $\pm$ 22%
	taxol 20.0 mg/kg IP 5X/WK	113 $\pm$ 22%
	mitomycin C 2.4 mg/kg IP 1X/WK	149 $\pm$ 12%
	adriamycin 2.6 mg/kg IP 1X/WK	105 $\pm$ 25%
20	diketone 30 mg/kg IP 3X/WK (listed original application)	163 $\pm$ 6%
	iodoacylfulvene 20 mg/kg IP 3X/WK	120 $\pm$ 34%
25	p-hydroxybenzylfulvene 15 mg/kg IP 3X/WK	125 $\pm$ 16%
	p-hydroxybenzylfulvene 20 mg/kg IP 3X/WK	126 $\pm$ 22%
	6-hydroxymethylacylfulvene 10 mg/kg IP 3X/WK (2 animals alive, ? cured)--	> 204%
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Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

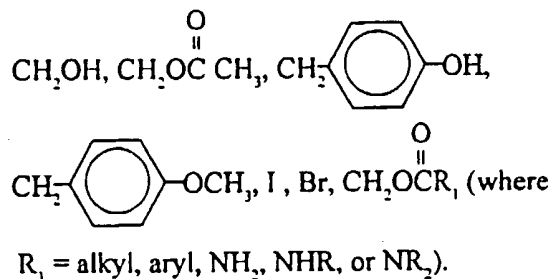


## WE CLAIM:

1. A method of inhibiting tumor growth in a subject comprising contacting the tumor with a therapeutic amount of an acylfulvene analog having the structure:



wherein the analog is capable of inhibiting tumor growth without recessive toxicity to the subject and wherein R is selected from the group consisting of:

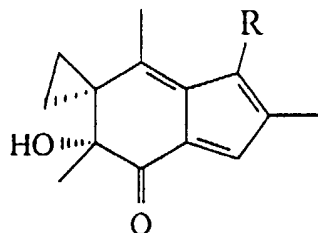


2. The method of claim 1 wherein the therapeutic amount is between 30 to 112,000  $\mu\text{g}$  per kg of body weight with intravenous administration.

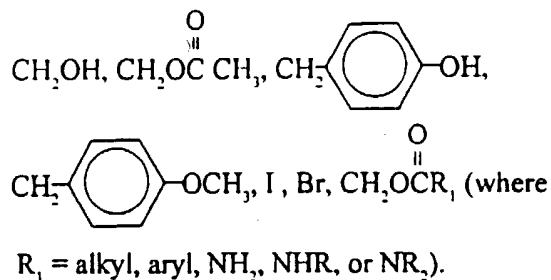
3. The method of claim 1 wherein the therapeutic amount is between 300 to 112,000  $\mu\text{g}$  per kg of body weight intraperitoneal.

4. The method of claim 1 wherein the analog is administered by any means selected from intravenous, oral, intraperitoneal, and inhalation.

5. A composition capable of binding to a tumor antigen comprising a reagent attached to an acylfulvene analog having the structure:



wherein R is selected from the group consisting of:



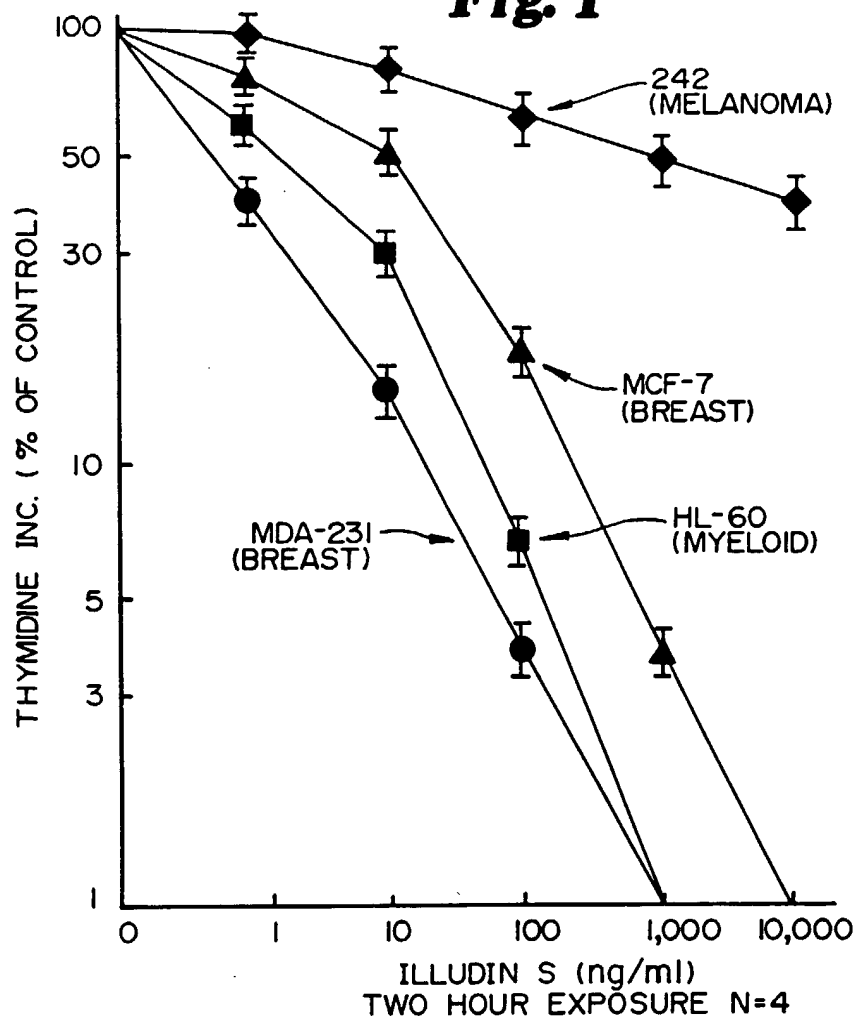
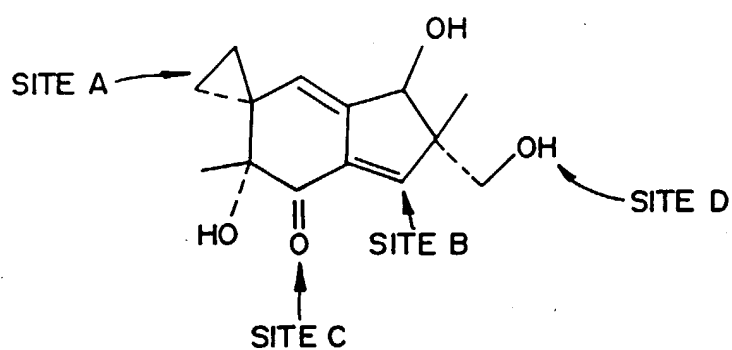
6. The complex of claim 5 wherein the reagent is an antibody.

7. A method of inhibiting tumor cell growth in a subject comprising contacting the tumor with the composition of claim 6.

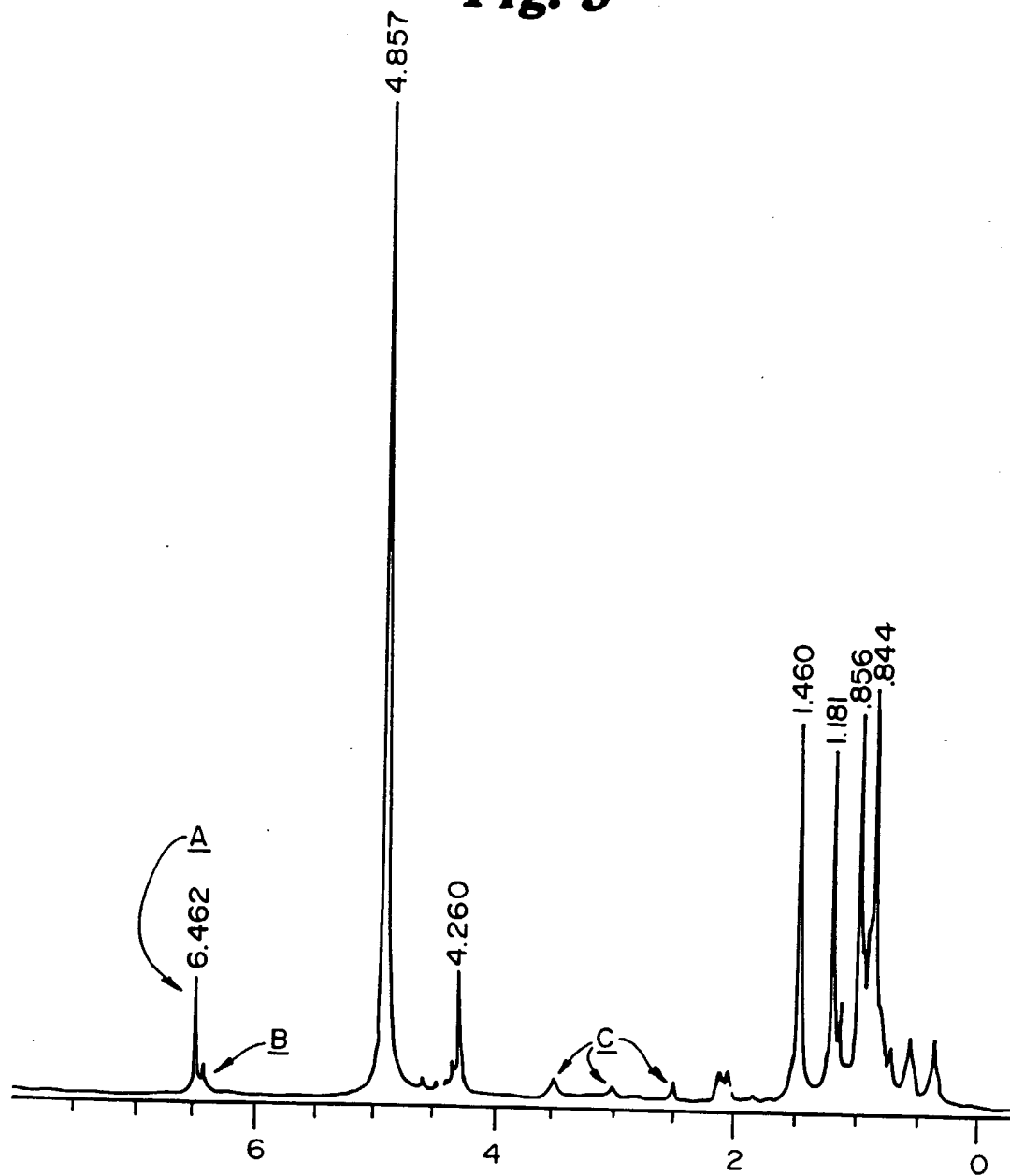
8. A therapeutic composition comprising 30 to 100 mg of the compound of claim 1 and a pharmaceutically acceptable carrier.

9. The method of claim 1, wherein the tumor cell is selected from the group consisting of myeloid, epidermoid, T-cell leukemia, and lung, ovarian and breast carcinoma.

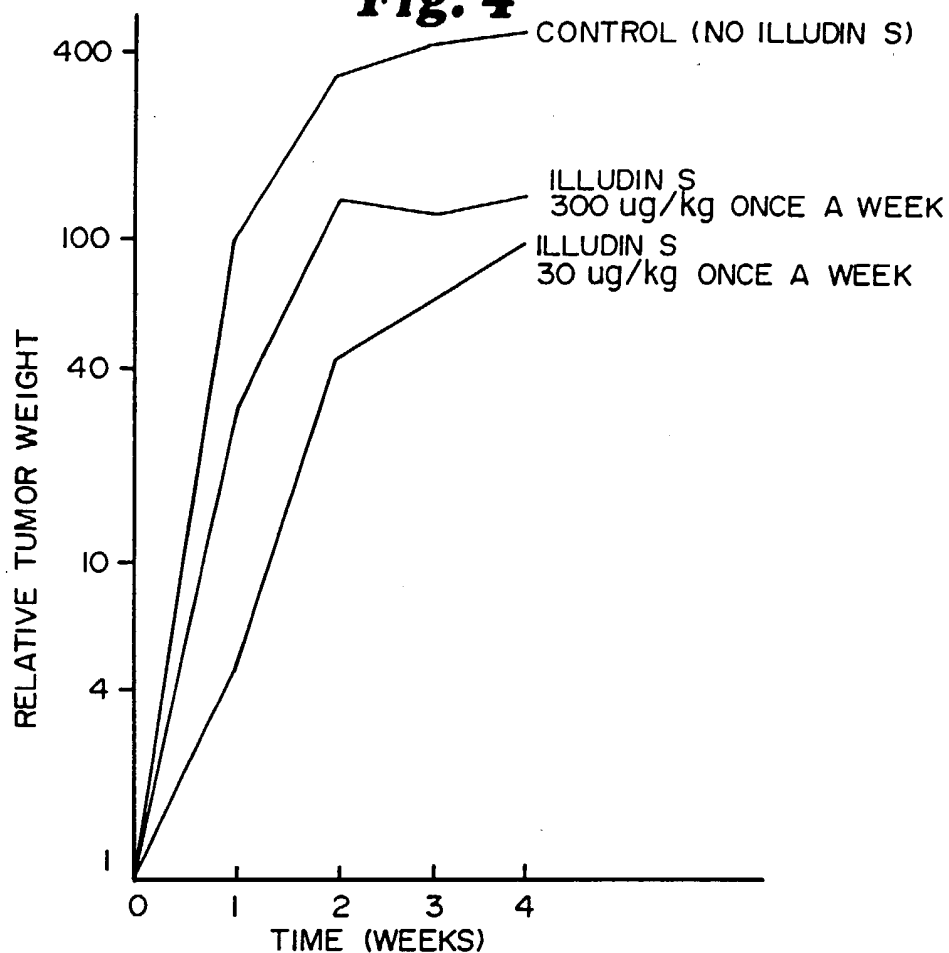
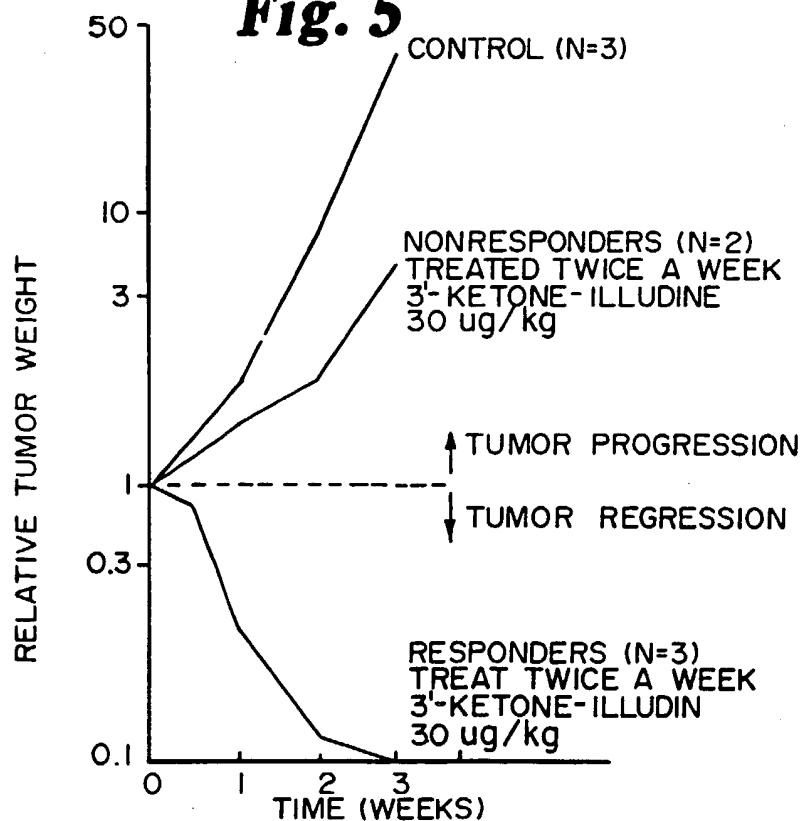
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**Fig. 1****Fig. 2**

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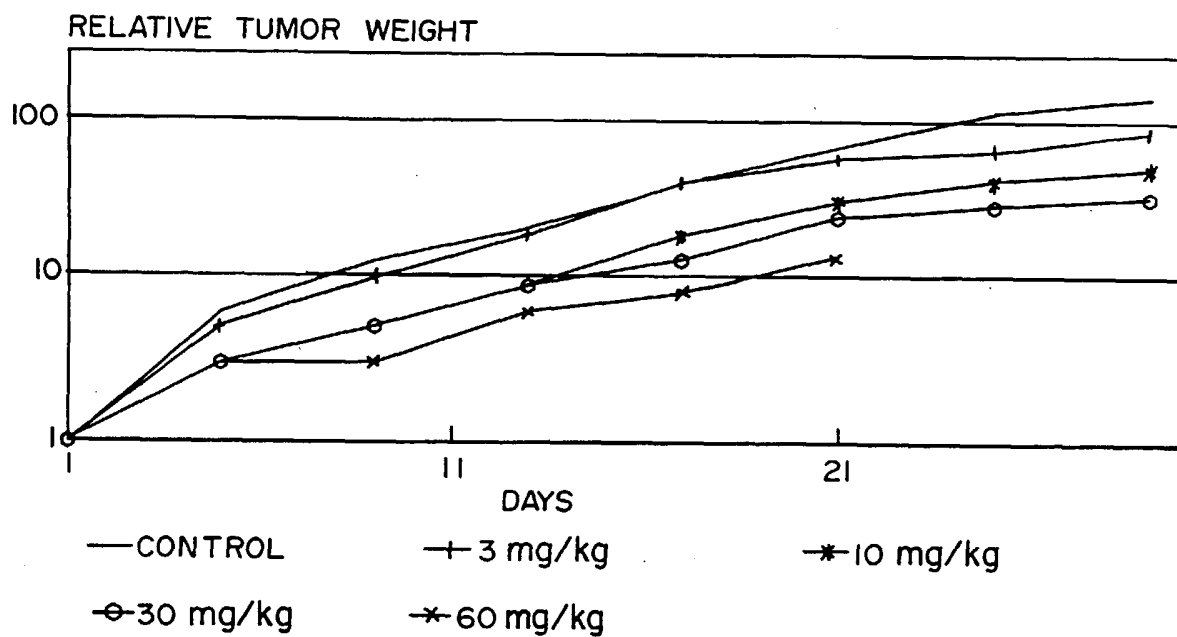
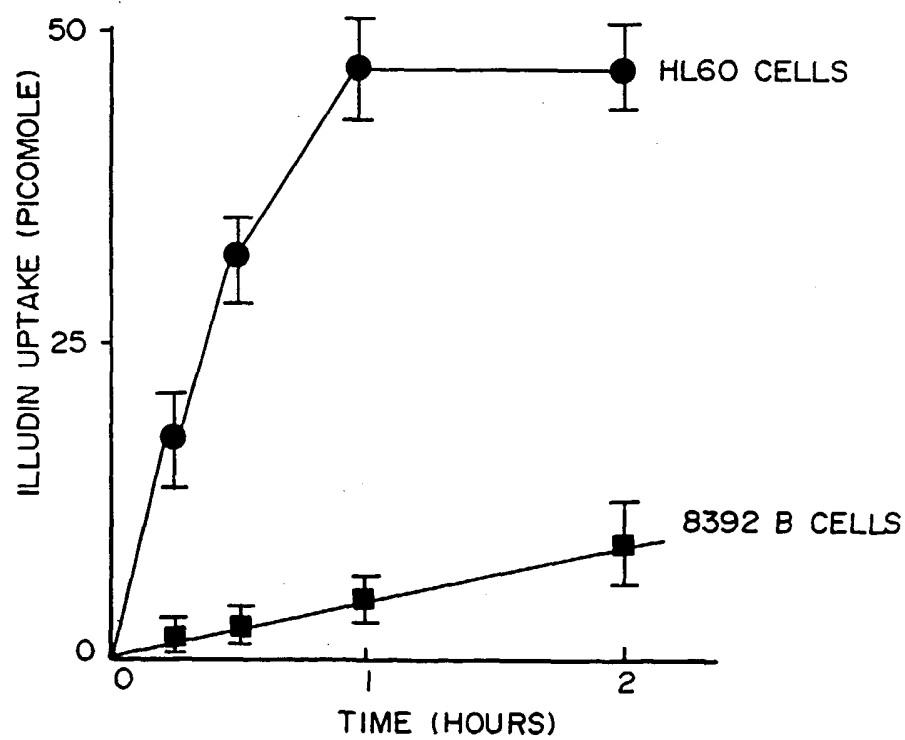
**Fig. 3**

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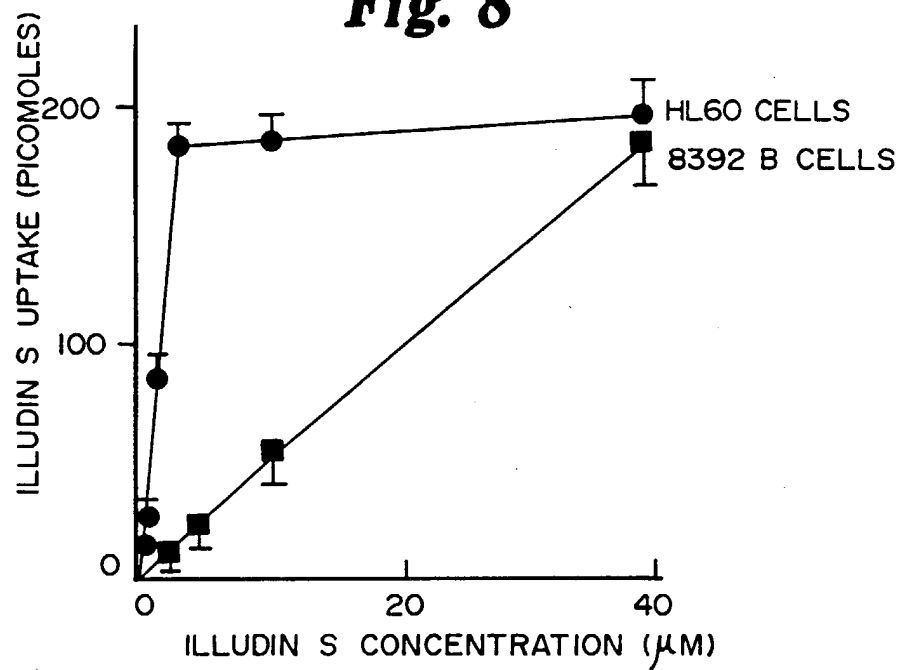
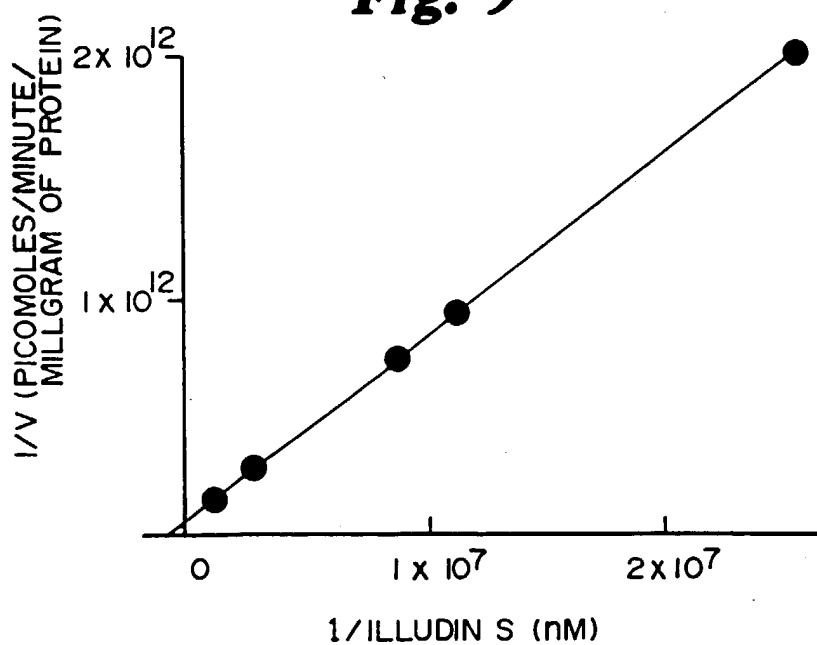
**Fig. 4****Fig. 5**

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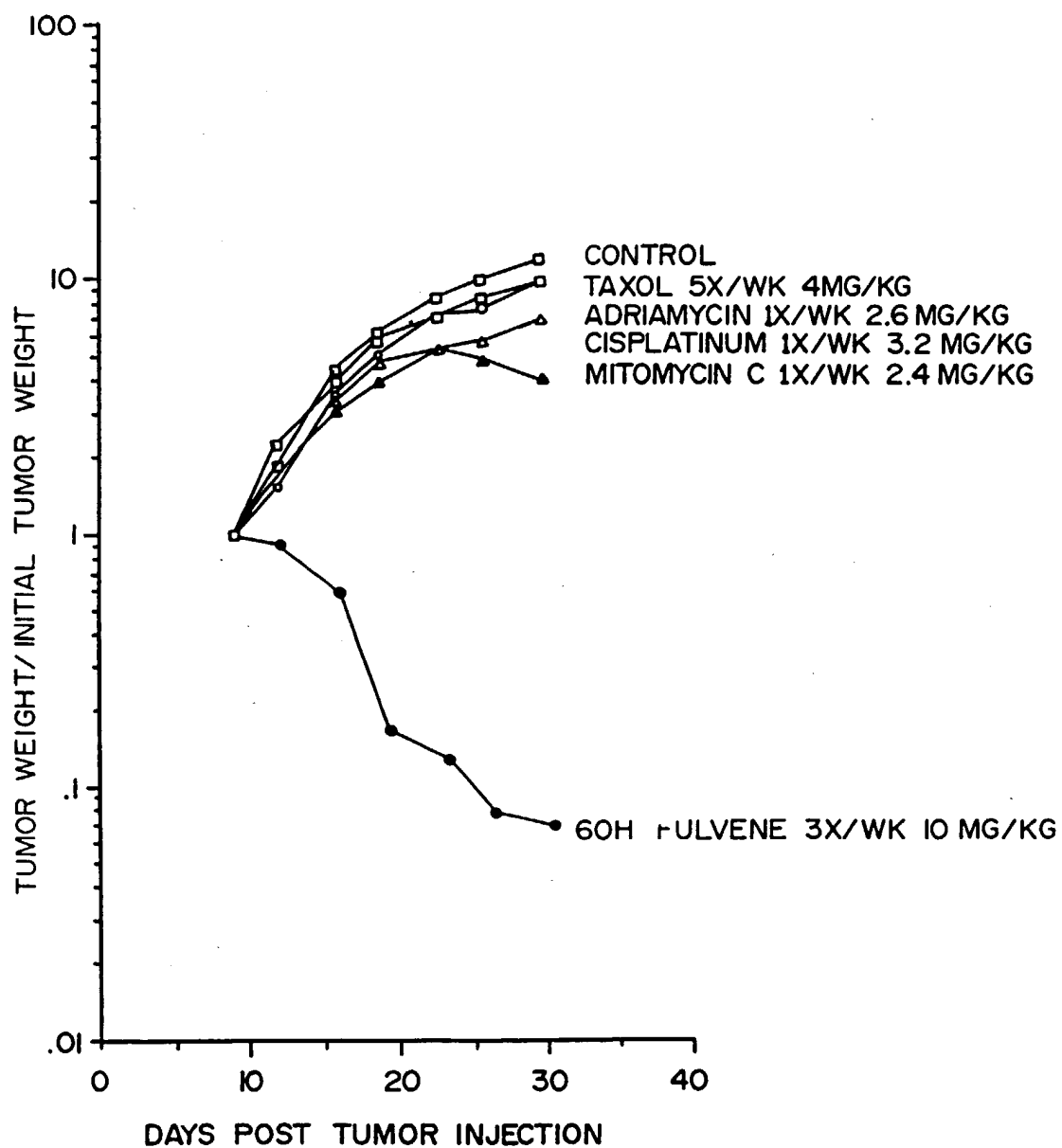
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**Fig. 6****Fig. 7**

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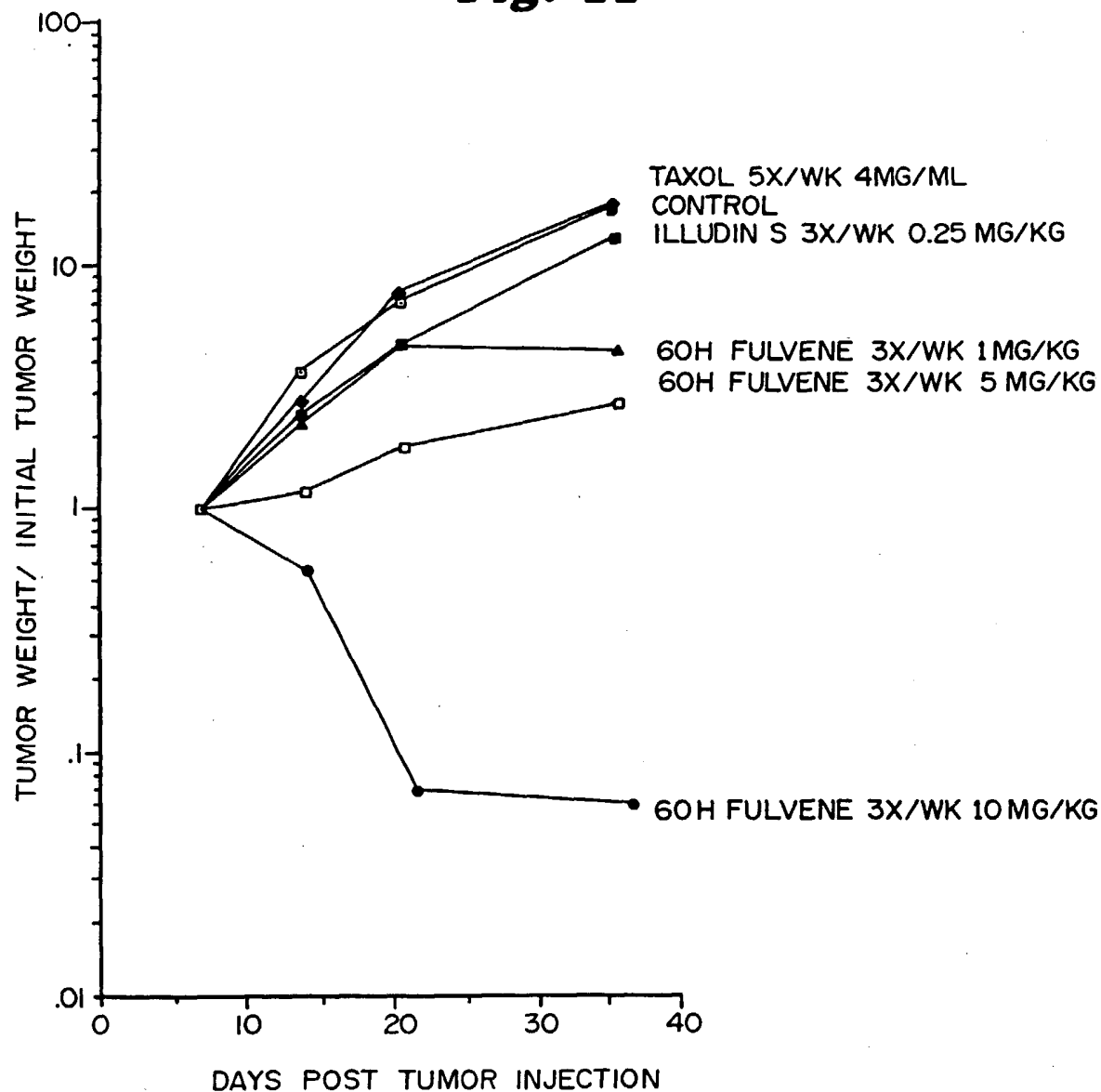
**Fig. 8****Fig. 9**

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**Fig. 10**



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**Fig. 11**

T/US 94/01232

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07C49/737 A61K31/12 A61K31/215 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 04754 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 18 April 1991 see page 6; claims 2,4; tables 8,9,10,12 ---	1-9
Y	TETRAHEDRON vol. 45, no. 17, 1989 pages 5433 - 5440 T.C. MCMORRIS ET AL. 'Structure and reactivity of illudins' see page 5437, last paragraph - page 5438, paragraph 1 -----	1-9

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

9 June 1994

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